

# Dose-Response Relationship to Inhaled Endotoxin in Normal Subjects

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Exposure to endotoxin and to its purified derivative lipopolysaccharide (LPS) is related to several occupational pulmonary diseases and to severe domestic asthma. An inhalation of a given dose of pure LPS produces both a systemic and a bronchial inflammatory response. Information on the dose-response relationship to inhaled LPS in normal subjects is a prerequisite to define the safety threshold of exposure. In the present study, the clinical and inflammatory responses to rising doses of inhaled LPS was evaluated. Nine normal volunteers were challenged weekly by inhalation with saline, 0.5, 5, and 50  $\mu\text{g}$  LPS (*Escherichia coli*). The response determinators are the clinical symptoms, fever,  $\text{FEV}_1$ , blood polymorphonuclear neutrophils (PMNs) with their level of activation (measured by luminol enhanced-chemiluminescence), and both the blood and the urine concentrations of the C-reactive protein (CRP). To assess the bronchial inflammatory response, an induced sputum was obtained 6 h after each dose of LPS, and the total and differential cell counts as well as the MPO, ECP, and  $\text{TNF-}\alpha$  concentrations were measured. Compared with the saline, an inhalation of 0.5  $\mu\text{g}$  LPS induces a significant decrease in the PMN luminol-enhanced chemiluminescence ( $p < 0.01$ ), which could reflect a process of margination and/or extravascular sequestration of activated PMN. Inhalation of 5  $\mu\text{g}$  LPS is associated with a significant rise in blood CRP ( $p < 0.01$ ) and PMNs ( $p < 0.001$ ) and in sputum PMNs ( $p < 0.05$ ), monocytes ( $p < 0.05$ ), and MPO ( $p < 0.05$ ). Inhalation of 50  $\mu\text{g}$  LPS was characterized by a significant increase in temperature ( $p < 0.01$ ), blood PMNs ( $p < 0.001$ ), blood and urine CRP ( $p < 0.01$  and  $< 0.01$ ), and sputum PMNs ( $p < 0.001$ ), monocytes ( $p < 0.05$ ), lymphocytes ( $p < 0.05$ ), MPO ( $p < 0.01$ ),  $\text{TNF-}\alpha$  ( $p < 0.01$ ), and ECP ( $p < 0.01$ ) while five subjects develop symptoms. In normal subjects, the response to inhaled LPS is dose-related, the most sensitive markers of LPS-induced inflammation being the blood PMNs count with their level of activation, the blood CRP concentration, and the sputum PMNs count. The no-response threshold to an acute inhalation of LPS is less than 0.5  $\mu\text{g}$ . **Michel O, Nagy A-M, Schroeven M, Duchateau J, Nève J, Fondu P, Sergysls R. Dose-response relationship to inhaled endotoxin in normal subjects.**

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Endotoxin and its purified derivative lipopolysaccharide (LPS) are gram-negative bacterial constituents continuously shed into the surrounding environment. These highly potent proinflammatory substances are ubiquitous in the environment. In humans, an acute inhalation of pure endotoxin (or dust containing endotoxin) induces a blood and lung inflammatory reaction in which neutrophils and macrophages are involved (1, 2) and clinical symptoms, including fever and shaking chills (3, 4), a lung function response characterized by

bronchoconstriction and change in the level of nonspecific bronchial hyperresponsiveness (BHR) (3-6). The chronic exposure of endotoxin measured in the dust from occupational and domestic settings has been related to both the risk of developing nonatopic chronic obstructive pulmonary diseases (7, 8) and the severity of domestic asthma (9). Thus, there is now a lot of evidence that environmental endotoxin is related to several lung diseases and that there is a need to control endotoxin exposure to prevent the development of these diseases.

Information on the dose-response relationship is a prerequisite to define the no-response threshold of exposure that should be considered as the safety concentration of endotoxin contamination in airborne dust. The time-related response has been intensively investigated by measuring several systemic (blood level) and local (lung function and bronchial lavage) inflammatory parameters after inhalation of a given dose of endotoxin (1-5), but available data on the dose-response relationship are less extensive. Although, Castellán and coworkers (6) and Rylander and colleagues (3, 10) have reported the relation between endotoxin levels and the decrease in  $\text{FEV}_1$

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TABLE 1  
CHARACTERISTICS OF THE SUBJECTS

Subject No.	Main Characteristics at Baseline					Clinical Response to 50 µg Inhaled LPS				
	Sex	Age (yr)	FEV <sub>1</sub> * (L)	FEV <sub>1</sub> <sup>†</sup> (L)	Weight (kg)	Malaise	Myalgia	Shivers	Fatigue	Time of Onset (h)
1	M	24	3.9	4.5	68	—	+	—	—	12
2	M	25	4.7	4.8	89	—	—	—	—	—
3	F	45	2.8	2.7	58	—	+	+	+	8
4	F	43	2.6	2.6	65	—	—	—	—	—
5	F	43	3.0	2.7	70	—	—	—	+	8
6	M	24	5.7	5.0	85	+	+	—	—	12
7	M	28	4.5	4.5	79	+	—	—	—	8
8	M	31	3.4	4.3	74	—	—	—	—	—
9	F	50	2.7	2.9	79	—	—	—	—	—

\* At baseline.

<sup>†</sup> Predicted value.

among selected normal subjects (6), active cotton workers (10), and healthy subjects (3), the dose-response relationship in a given subject, measured by sensitive local and systemic markers of inflammation, has not been established.

A group of normal volunteer subjects was challenged by inhalation of increasing doses of pure endotoxin while effect determinators were clinical symptoms, fever, change in lung function, BHR, blood, urine, and bronchial (by using the technique of induced sputum) inflammatory markers. The no-response threshold was then defined for each parameter.

## METHODS

### Subjects (Table 1)

In a group of 12 normal subjects, nine volunteers were preselected for their ability to produce an adequate sputum sample induced by inhalation of a hypertonic saline solution (*see below*). A sputum sample was considered as adequate if the weight of the sputum was at least 2 g and if, on differential cell counting, it contained less than 50% squamous epithelial cells (11). None of the subjects had a recent and/or a previous medical history, none was a current or ex-smoker or was receiving medication. On the basis of positive skin prick tests for the most frequent airborne allergens, five subjects were considered atopic and four nonatopic. There were four women and five men with a mean age of  $35 \pm 3.5$  yr (range, 24 to 50 yr), and their mean basal FEV<sub>1</sub> was  $98.4 \pm 3.5\%$  (range, 79.1 to 114.1%) of the predicted value (12). None was characterized by a significant nonspecific bronchial hyperreactivity (i.e., a PD<sub>20</sub>FEV<sub>1</sub> < 200 µg histamine; for methodology, *see below*). Written informed consent was obtained from each subject, and the protocol was approved by the Ethical Committee of the Hospital.

### General Study Design (Figure 1)

At 1-wk intervals, in a single-blind fashion, each subject was submitted to a bronchial challenge with sterile saline and increasing (0.5, 5, and 50 µg) inhaled doses of pure LPS (from *Escherichia coli* serotype 026:B6 extracted by TCA precipitation and gel filtration chromatography, ref. L-2654, lot 43H40491) (Sigma Chemical, St. Louis, MO) using a method previously described in detail (12). All the inhaled solutions were administered by a Mefar dosimeter MB3 (Mefar, Brescia, Italy). This dosimeter produces a calibrated aerosol of 9 µl at each slow inhalation starting from FRC to just below TLC, the nebulizer weight loss per actuation being measured for calibration control (13). The jet nebulizer generated heterodisperse droplets with a mass median aerodynamic diameter of 1 to 4 µm, the variation within this range having little effect on the response (13). The highest dose of LPS (50 µg) corresponded to the threshold for inducing clinical symptoms suggested by Rylander and colleagues (3), whereas in our experience 20 µg inhaled LPS does not induce any symptoms (2). To en-

sure the safety of the subjects, the solutions of LPS were given to each subject, in the same order, from the lower to the highest dose. Clinical symptoms, fever, and lung function were recorded before and 1, 6, 8, and 24 h after each challenge. By doing so, subjects developing significant symptoms after 0.5 or 5 µg LPS could be excluded from the study. The level of BHR was evaluated by measuring PD<sub>20</sub>FEV<sub>1</sub> for histamine at 8 h, and a sample of induced-sputum was obtained at 6 h and blood samples were obtained before and 6 h after (just before sputum) the bronchial challenges. At 24 h, urine and blood samples were also obtained. Eight hours after the challenge, the subjects were authorized to leave the hospital with the possibility of telephone contact with a physician involved in the study. No subject dropped out of the study at any time.

### Lung Function Measurements

Lung function parameters were measured with a constant-volume computerized plethysmography (Bodyscreen II; E. Jaeger, Würzburg, Germany), and the FEV<sub>1</sub> was computed from integration of the maximal expiratory flow-volume curves. The level of nonspecific bronchial hyperresponsiveness was assessed by histamine challenge test as previously described (12). The cumulative provocative dose of histamine inducing a 20% fall in FEV<sub>1</sub> (PD<sub>20</sub>FEV<sub>1</sub>) was calculated from the dose-response curve. Because several of the subjects did not achieve a decrease of 20% in their FEV<sub>1</sub>, the results are expressed as the maximal decrease in FEV<sub>1</sub> (expressed as a percentage of the baseline value) measured after inhalation of the last dose of histamine (similar for each study day in a given subject).

### Blood Parameters

Total white blood cells (WBC) and differential proportion were counted using an automated method (H1 counter; Technicon Instru-

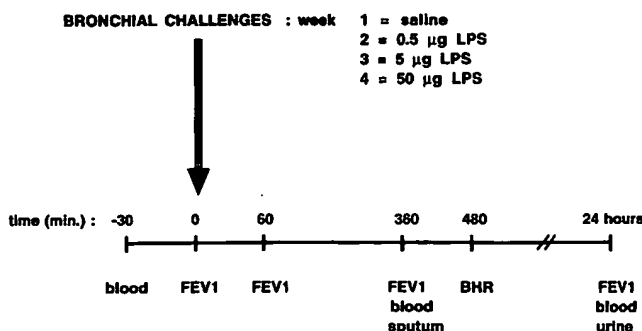


Figure 1. General study design. BHR = bronchial hyperresponsiveness (i.e., the maximal FEV<sub>1</sub> decrease after a given dose of histamine in each subject).

ments, Tarrytown, NY). After centrifugation the plasma was stored at  $-20^{\circ}\text{C}$ . The blood and urine CRP concentrations were measured by using a two-site monoclonal antibody enzyme-linked immunoassay (antibodies and calibrator from Dako Ltd, High Wycombe, UK), the limit of sensitivity being  $0.5\text{ }\mu\text{g/L}$ .

#### Luminol-enhanced Chemiluminescence of Blood PMN

The PMNs were isolated at room temperature from heparinized blood by using a modified Ficoll-Paque (Pharmacia, Uppsala, Sweden) procedure (14). The plasma layers were removed by centrifugation. The resulting rich layer of PMNs (buffy coat) was diluted in three volumes of PBS (pH, 7.2) and layered on two volumes of Ficoll-Paque. After centrifugation at  $1,000\text{ g}$  for 30 min, mononuclear cells and Ficoll-Paque layers were discarded. The remaining erythrocytes were lysed in a hypotonic solution containing  $0.15\text{ M NH}_4\text{Cl}$ ,  $0.1\text{ mM EDTA}$  (pH, 7.4) and removed by centrifugation at  $400\text{ g}$ . The resulting leukocyte pellet was washed in Hank's balanced salt solution without phenol red (HBSS), without  $\text{Ca}^{2+}$ , and without  $\text{Mg}^{2+}$  (Gibco Europe GmbH, Karlsruhe, Germany) and resuspended in HBSS at  $10^7$  cells/ml. Cell viability assessed by trypan blue exclusion dye always exceeded 98%, and the purity of PMNs was  $>96\%$  in all cases.

The reactive oxygen species (ROS)-induced chemiluminescence (CL) of PMNs was measured in duplicate in a Model 1251 BioOrbit luminometer (BioOrbit, Turku, Finland) using luminol to enhance the luminescence of the whole set of ROS (15). Luminol (Sigma) was dissolved at  $10^{-2}\text{ M}$  in dimethylsulfoxide and further diluted at  $10^{-6}\text{ M}$  in HBSS prior to use. The total volume of the reaction mixture was  $1\text{ ml}$ , with each sample containing  $1.25 \times 10^6$  cells. The PMNs ( $250\text{ }\mu\text{l}$ ) were first incubated at  $37^{\circ}\text{C}$  with  $10^{-6}\text{ M}$  luminol ( $250\text{ }\mu\text{l}$ ) and the resulting spontaneous light emission was continuously measured for 1 h. The PMNs suspension was then stimulated with  $500\text{ }\mu\text{l}$  of opsonized zymosan (from *Saccharomyces cerevisiae*, 1 yeast for 10 PMNs) and CL was further recorded for 30 min. The intensity of PMN-CL was determined by computing the area under the curve (AUC) for the first 20 min of incubation (millivolts  $\cdot 20\text{ min}/1.250 \times 10^6$  cells). The intra-assay coefficient of variation (CV) was  $<7\%$  ( $n = 100$ ).

#### Induction and Analysis of Sputum

Induced sputum was obtained using the method described by Pin and coworkers (16) adapted for normal subjects. In particular, the subjects did not inhale  $\beta$ -agonist before the procedure, and the concentration of saline was not increased at 10-min intervals. The hypertonic sterile saline (5%) was nebulized with an ultrasonic nebulizer (Fisoneb; Karapharm, Marseille, France) and was inhaled for a 30-min period. Ten minutes after the start of nebulization and every 5 min thereafter, subjects were asked to rinse their mouths with water and to try to cough sputum directly in a plastic box. No subject had a decrease of  $\text{FEV}_1$  of 10% or more at the end of the procedure.

After the volume of the sputum was recorded, an equal volume of dithiotriitol 10% (Sputolysin; Berhing Diagnostics, Somerville, NJ) was added to the sample of sputum and mixed by vortex and shaking water, both at  $37^{\circ}\text{C}$  for 15 min, to suspend the plugs for total cell counts. Differential cell counts of squamous and bronchial epithelial cells and of leukocytes, neutrophils, eosinophils, lymphocytes, macrophages, and monocytes were performed by counting nucleated cells on each of two slides fixed with methanol and stained with May-Grünwald-Giemsa. The total and differential cell counts were evaluated by two independent experienced readers, and their means were calculated. The supernatants were frozen at  $-20^{\circ}\text{C}$  for later analysis.

TABLE 2  
BASELINE VALUES OF LUNG FUNCTION, FEVER, AND  
BLOOD MARKERS FOR EACH STUDY DAY\*

	Saline	LPS 0.5 $\mu\text{g}$	LPS 5 $\mu\text{g}$	LPS 50 $\mu\text{g}$
$\text{FEV}_1$ , L	3.72 (0.36)	3.72 (0.33)	3.68 (0.37)	3.71 (0.36)
Temperature, $^{\circ}\text{C}$	36.4 (0.1)	36.4 (0.1)	36.5 (0.1)	36.4 (0.1)
PMNs, cells/ $\mu\text{l}$	2,680 (250)	2,920 (240)	3,070 (240)	2,710 (280)
CRP, mg/L	1.5 (0.4)	3.0 (2.2)	3.1 (2.3)	2.0 (1.1)

\* Values are means with SE shown in parentheses.

Several soluble markers involved in the monocytes-neutrophils activation ( $\text{TNF-}\alpha$ , IL-8, MPO) were evaluated in the induced sputum. The  $\text{TNF-}\alpha$  and IL-8 concentrations were measured with a specific immunoenzymatic assay ( $\text{TNF-}\alpha$ -easia and IL-8-easia; Medgenix Diagnostics, Belgium), the limits of sensitivity and the intra-assay CV being  $3\text{ pg/ml}$  and  $5.2\%$  for  $\text{TNF-}\alpha$  and  $0.7\text{ pg/ml}$  and  $4.9\%$  for IL-8. The myeloperoxidase (MPO) concentration was evaluated by a RIA commercial kit (MPO RIA; Pharmacia, Uppsala, Sweden) with a sensitivity of  $8\text{ ng/ml}$  and a  $8.3\%$  intra-assay CV. The eosinophil cationic protein (ECP) was measured with the ECP RIA commercial kit (Pharmacia).

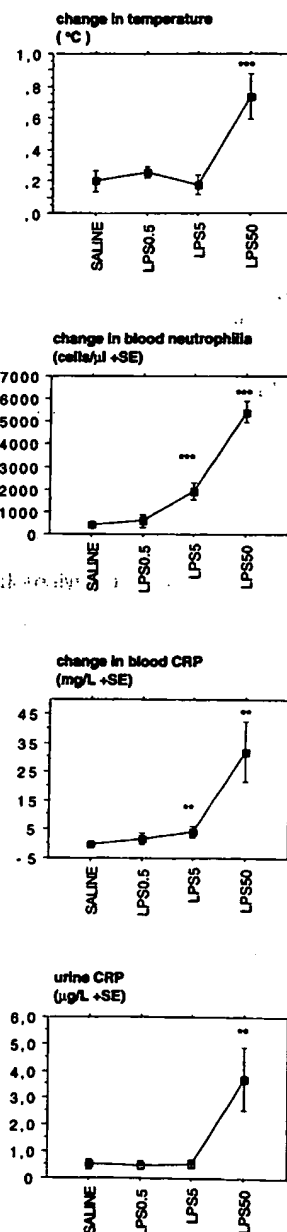


Figure 2. The systemic response after saline, 0.5, 5, and  $50\text{ }\mu\text{g}$  LPS inhalation. Results are means ( $\pm$  SE) of the rise in temperature and blood absolute neutrophilia 6 h after the challenges ( $F_{24}^3$  test =  $8.53$ ,  $p < 0.001$  and  $F_{24}^3$  test =  $83.00$ ,  $p < 0.001$ , respectively) and in blood and urine CRP 24 h after the challenges ( $F_{24}^3$  test =  $10.15$ ,  $p < 0.001$  and  $F_{24}^3$  test =  $8.24$ ,  $p < 0.001$ , respectively). \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

Concentration of albumin in sputum was determined as markers of plasma exudation in the airways. Albumin concentration was measured by automatic nephelometry (BNA; Behring, Brussels, Belgium) after immunoprecipitation with a specific antibody (rabbit antihuman albumin; Behring), the limit of sensitivity being 1 mg/dl and the intra-assay CV less than 5%.

### Statistics

All the results are calculated as mean  $\pm$  1 SE. Concentrations in mediators, total and differential cells in sputum, and CRP in urine are expressed in absolute values, whereas temperature, blood neutrophilia, and CRP concentrations are expressed as changes compared to time 0. A two-way analysis of variance (ANOVA) was performed on each parameter, taking into consideration the factors subject and treatment (i.e., saline, 0.5, 5, and 50  $\mu$ g LPS). When the F-test was significant, the values of the parameters were compared with the basal value (i.e., after saline) for each dose of endotoxin by a modified *t* test (using the mean square of residual variance), and *p* was calculated with the Bonferroni correlation (17). A *p* value  $< 0.05$  was considered as significant.

### RESULTS

The baseline values of FEV<sub>1</sub>, temperature, and blood markers were not significantly different for each of the four study days (Table 2).

Although 0.5 or 5  $\mu$ g inhaled LPS didn't induce any clinical symptoms and/or fever in these normal subjects, 50  $\mu$ g LPS was associated with a significant increase ( $0.7 \pm 0.1^\circ\text{C}$ ) of the body temperature, whereas in five subjects, acute systemic symptoms were observed 8 to 12 h after the challenge, lasting no more than 4 h (Figure 2 and Table 1).

The dose-related increase in both blood PMN count and CRP concentration was significant after 5  $\mu$ g, and it was reinforced after inhalation of 50  $\mu$ g LPS (Figure 2). The blood CRP induced with 5  $\mu$ g LPS was predictive for its response with 50  $\mu$ g since both responses are highly significantly related (Figure 3); the same but less significant relation was observed for blood neutrophilia (Figure 3). Urine excretion of CRP can be detected when the subjects are challenged with 50  $\mu$ g LPS (Figure 2).

The changes in PMN-CL were statistically significant, the dose-response relationship being biphasic. Indeed, compared with the saline, an inhalation of 0.5  $\mu$ g LPS induced a decrease in the level of blood PMN-CR, whereas 5 and 50  $\mu$ g of inhaled LPS were associated with a rise in PMN-CL (Figure 4). A significant dose-response relationship was observed for PMN-CL after 0.5, 5, and 50  $\mu$ g LPS.

A slight (but not significant) change in the mean FEV<sub>1</sub> was seen after 50  $\mu$ g LPS (mean FEV<sub>1</sub>,  $97.4 \pm 1.3\%$  of the basal FEV<sub>1</sub>) compared with the saline (mean FEV<sub>1</sub>,  $99.2 \pm 1.1\%$  of the basal FEV<sub>1</sub>). The degree of nonspecific BHR rises 8 h after bronchial challenge with 50  $\mu$ g compared with saline, 0.5 and 5  $\mu$ g LPS, but this effect was not statistically significant (histamine-induced decrease in FEV<sub>1</sub>,  $14.7 \pm 4.1$  versus  $9.8 \pm 2.6\%$ , 8 h after 50  $\mu$ g LPS or saline, respectively). Three subjects (Subjects 2, 5, and 7) had a significant rise in their degree of BHR, characterized by a decrease of PD<sub>20</sub>FEV<sub>1</sub> of more than one histamine dilution.

The technique of induced sputum doesn't produce any significant side effect and/or change of more than 10% in FEV<sub>1</sub>. After saline inhalation (Week 1) induced sputum was characterized by a percentage of squamous cells of less than 40% (range, 6 to 38%). The sputum of Subject 6 sampled during Week 4 was excluded from the analysis since it is characterized by 90% squamous cells.

The LPS inhalation was associated with a dose-dependent significant increase in the total and differential (neutrophils,

lymphocytes, and monocytes) cell count in sputum (Figure 5). A neutrophilic infiltration in sputum was significant after 5  $\mu$ g LPS inhalation compared with saline (mean PMN in sputum,  $7.4 \pm 2.1 \times 10^5$  versus  $3.7 \pm 0.9 \times 10^5$  cells/ml, respectively; *p*  $< 0.05$ ) and this response was significantly reinforced after the highest tested dose (50  $\mu$ g) of inhaled LPS. The inhalation of LPS did not induce significant changes in the count of eosinophils, macrophages, and squamous or bronchial epithelial cells in sputum.

The concentration of MPO in sputum rose significantly after 5  $\mu$ g LPS inhalation (Figure 6). The dose-response relationship was similar for the neutrophil count in sputum, the ratio MPO/PMN being unchanged after inhaled LPS ( $94 \pm 20$   $\mu$ g MPO/ $10^3$  PMN versus  $62 \pm 4$   $\mu$ g MPO/ $10^3$  PMN after saline and LPS 50  $\mu$ g, respectively; *p* = NS). Although the eosinophils count didn't modify significantly, the concentration of

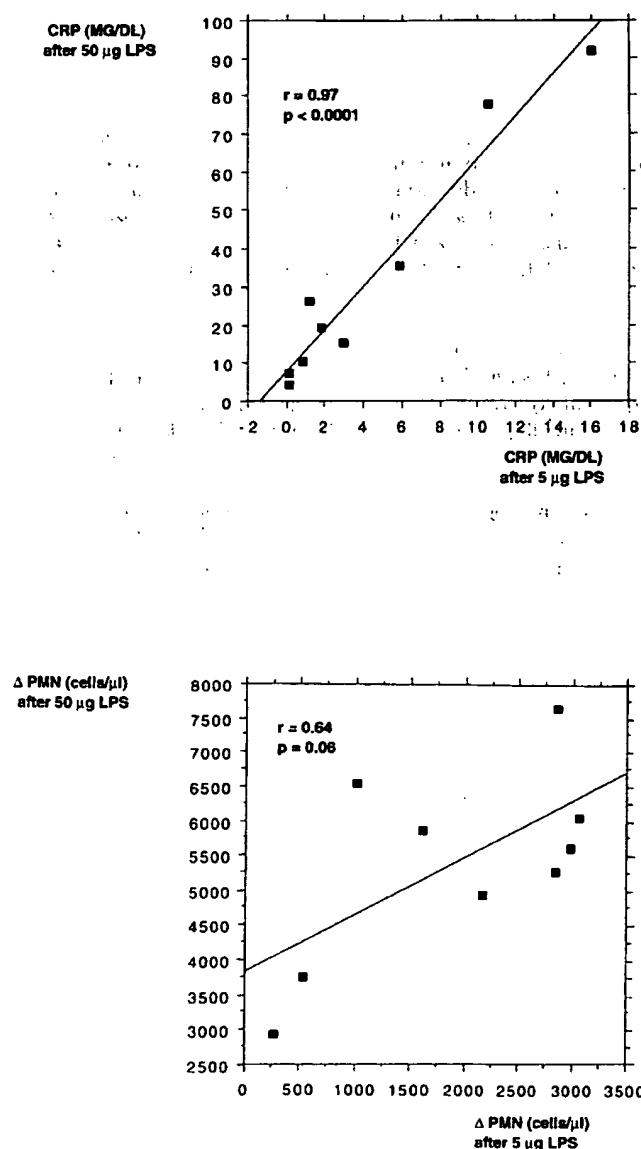
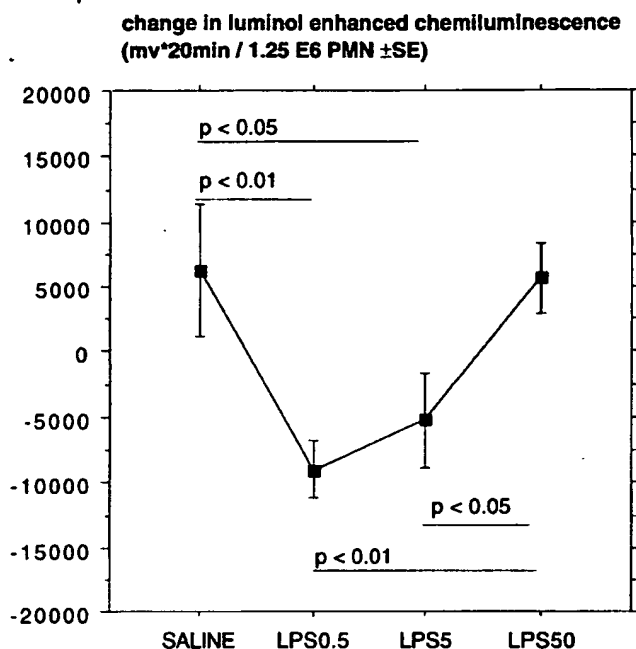


Figure 3. (Top panel). Relationship between the blood CRP concentration measured 24 h after an inhalation of 5  $\mu$ g LPS and after 50  $\mu$ g LPS. (Bottom panel) Relationship between the increases in PMN blood count after 5 and 50  $\mu$ g inhaled LPS.



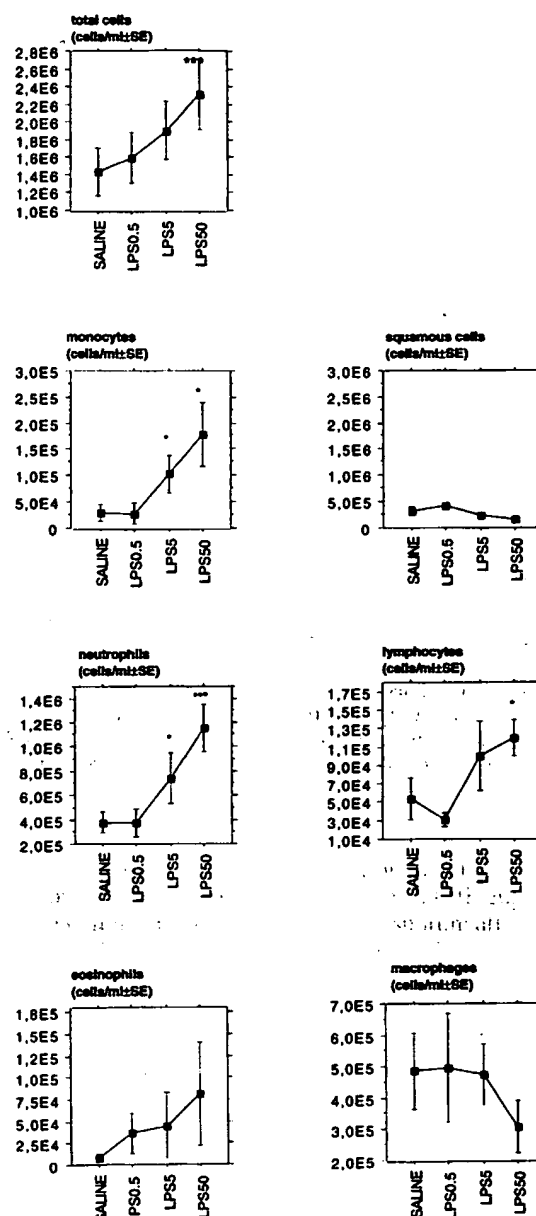
**Figure 4.** The degree of PMN stimulability evaluated by the AUC of their luminol-enhanced chemiluminescence (CL) measured for 20 min after *in vitro* stimulation. Changes in PMN CL were significant after the challenges ( $F^3_{21}$  test = 4.68,  $p < 0.01$ ).

ECP in sputum was increased after 50  $\mu$ g LPS ( $47 \pm 8$   $\mu$ g/ml compared with  $28 \pm 6$  after saline;  $p < 0.05$  [Figure 6]). There was a significant increase of TNF- $\alpha$  in sputum after 50  $\mu$ g LPS, characterized by a mean concentration of  $63 \pm 14$  ng/L (Figure 6). The concentrations of IL-8 and albumin did not change after LPS inhalation.

## DISCUSSION

This study indicated that in normal subjects, the response (i.e., clinical symptoms, fever, and bronchial/systemic inflammatory markers) to LPS is related to the inhaled dose.

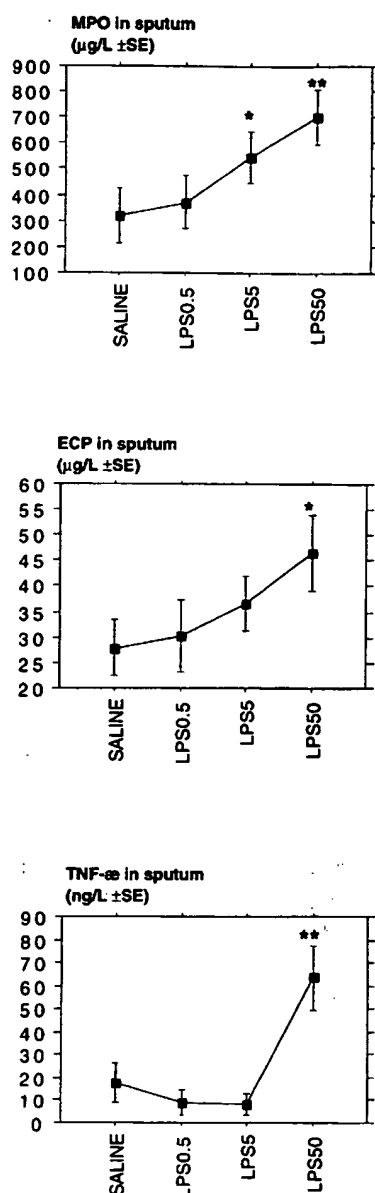
The maximal level of LPS exposure (i.e., 50  $\mu$ g) was potentially able to induce acute symptoms, fever, and change in lung function with dyspnea (1, 3, 12). Taking this into account, as well as the subject-dependent high variability of amplitude of the response to LPS (18), to assume the safety of the subjects, the bronchial challenges were performed in single-blind, the doses of LPS being increased weekly in the same order. This experimental design could have introduced a bias since each previous LPS inhalation, as well as the saline hypertonic aerosol (required to induce sputum), could modify the response to the next LPS dose. Nevertheless, the similar basal values of the parameters measured just before each bronchial challenge suggests that the subjects had recovered their basal status. In other studies, retrieval of the LPS ( $\geq 20$   $\mu$ g) response was obtained in less than 7 d (2, 5), whereas on the other hand, the intrasubject reproducibility of the cell count in weekly induced sputum was highly significant (19). A possible effect of induction of tolerance to LPS on the results was also quite improbable in the present study since it is well known that the early-phase tolerance develops within several hours, lasting no more than 2 d, and the late-phase tolerance occurs after several weeks since it requires specific antibody production (20).



**Figure 5.** The mean cell count in sputum after saline, 0.5, 5, and 50  $\mu$ g LPS inhalation: total cells ( $F^3_{21}$  test = 5.81,  $p < 0.01$ ); monocytes ( $F^3_{21}$  test = 5.74,  $p < 0.01$ ); squamous cells ( $F^3_{21}$  test = 2.25,  $p = \text{NS}$ ); neutrophils ( $F^3_{21}$  test = 8.90,  $p < 0.001$ ); lymphocytes ( $F^3_{21}$  test = 4.56,  $p < 0.01$ ); eosinophils ( $F^3_{21}$  test = 1.31,  $p = \text{NS}$ ); macrophages ( $F^3_{21}$  test = 0.52,  $p = \text{NS}$ ). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

However, since three repeated LPS exposures were produced in the present study, it is not completely excluded that the sequence of exposures may have influenced the outcome.

Inhaled LPS 50  $\mu$ g elicited a significant change in BHR and in acute clinical symptoms in three and five subjects, respectively, suggesting a marked intrasubject variability of the response as yet reported after intravenously administered (21) or aerosolized LPS (19). Neither the change in temperature nor the lung function response were sensitive enough to measure the response since a significant rise in temperature requires an



**Figure 6.** Concentration of mediators in sputum after saline, 0.5, 5, and 50  $\mu$ g LPS inhalation ( $F_{3,21}$  test = 16.48,  $p < 0.001$ ; 4.82,  $p < 0.01$ ; 9.33,  $p < 0.001$  for myeloperoxidase (MPO), eosinophil cationic protein (ECP), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), respectively, \* $p < 0.05$ , \*\* $p < 0.01$ ).

exposure of 50  $\mu$ g LPS while the lung function remains unchanged. A significant change in temperature was observed after a high level of exposure to inhaled LPS (50  $\mu$ g, i.e., 675 ng/kg), whereas an intravenous dose of LPS of 4 ng/kg was sufficient for a similar response (21). This suggests that less than 1% of inhaled LPS should reach the vascular compartment to induce pyrexia in normal conditions. However, this systemic response might also be caused by local LPS activation of the airways' macrophages releasing IL-1, IL-6, and TNF- $\alpha$  involved in the fever process (21). Deleterious effects of smaller amounts of airborne endotoxin on lung function have been reported in several occupational or domestic settings (6–8, 10) that also could be explained by the simulta-

neous presence of other toxic agents in the dust. Moreover, the temporal pattern of exposure to LPS could play a major role in the dose-related clinical response. Indeed, Milton and coworkers (22) have reported that the prevalence of asthma is associated with subjects chronically exposed to LPS, whereas flulike respiratory illness is more frequent in subjects exposed intermittently to high levels of LPS.

Compared with the symptoms or change in lung function, the blood PMNs count and CRP concentration are more sensitive indices of an acute exposure to LPS. This observation is consistent with the dose-dependent pulmonary effect of inhaled LPS reported in the guinea pig (23). The rise in blood PMNs and CRP is related to the dose of inhaled LPS, the no-response threshold being included between 0.5 and 5  $\mu$ g. Taking into consideration the significant correlation of the responses after 5 and 50  $\mu$ g LPS, the blood PMNs and CRP measured after a subclinical dose of LPS (5  $\mu$ g) could be predictive of the response after 50  $\mu$ g LPS inhalation. This means that the rise in PMNs and/or CRP measured 6 and 24 h after inhalation of a limited dose of LPS (5  $\mu$ g) could characterize the general LPS responsiveness of a subject.

The CRP may be largely immunosuppressive to LPS-induced lung macrophage cytokine production (24) and, in a transgenic mice model, CRP inhibits alveolitis by reducing neutrophil influx and protein leakage into the lungs (25). These anti-inflammatory characteristics of CRP suggest that this LPS-induced acute-phase protein response could be related to the risk of developing lung diseases in subjects chronically exposed to LPS. The threshold value of 5  $\mu$ g inhaled LPS for a significant rise in blood CRP is consistent with other published data (2, 5, 25). For instance, Larsson and colleagues (26) have reported a significant increase in CRP and orosomucoid, another acute-phase protein, 24 h after inhalation of swine dust containing approximately 3  $\mu$ g LPS. Moreover, chronic exposure to dust containing endotoxin has also been associated with an increase in serum CRP concentrations, as in workers exposed to sewage dust (27).

In our volunteers, the CRP, measured by ELISA, was significantly excreted in the urine 24 h after an inhalation of 50  $\mu$ g LPS. As the subject-compliance for repeated blood samplings is generally poor in large epidemiologic studies, the measurement of the CRP concentration in urine could be an interesting marker of intense exposure to LPS in occupational settings.

The blood neutrophilia is significant 6 h after an inhalation of 5  $\mu$ g LPS and rises after 50  $\mu$ g LPS (mean change in blood neutrophilia,  $1,920 \pm 369$  and  $5,407 \pm 473$  PMN/ $\mu$ l, respectively). This dose-response relationship is consistent with published data. Indeed, in normal subjects, 20 to 70  $\mu$ g of inhaled LPS induces a rise of 4,000 or 6,000 PMNs/ $\mu$ l (2, 28). This neutrophilia is not due to a phenomenon of PMNs demargination from the small blood vessels but rather to an outpouring of cells from the bone marrow (29).

The present results do not associate this intense blood neutrophilia with significant change in their level of activation evaluated with the luminol enhanced chemiluminescence. More intriguing is the significant decrease in CL-PMN observed after an inhalation of a small dose (0.5  $\mu$ g) of LPS compared with the saline. Retention of PMNs in the pulmonary microvasculature is due to changes in their mechanical and adherence properties (30). Endotoxin stimulation decreases PMNs deformability, resulting in prolonged neutrophil sequestration in the pulmonary vasculature (30, 31). We hypothesize that 0.5  $\mu$ g inhaled LPS induces pulmonary retention of the active blood PMNs, leading to a decrease in CL-PMN. This also suggests that an exposure of the airways to small amounts of LPS produces a subtle biologic response.

Inflammation of the airways induced by inhaled LPS has been evaluated by cell and fluid phase markers in induced sputum and compared with the characteristics of samples obtained 6 h after saline inhalation. This technique is appropriate for investigating inflammatory processes involving PMNs in normal subjects (32). It is highly reproducible (19) and it has been validated in healthy subjects, asthmatics, and smokers by assessing the correlation of different markers in the sputum with clinical parameters (19). Compared with bronchoalveolar lavage or bronchial mucosal biopsies, induced sputum is a less invasive technique that can be repeated several times in the same subject. A significant increase in neutrophils and monocytes occurs in the induced sputum after an inhalation of 5  $\mu\text{g}$  LPS and is reinforced after 50  $\mu\text{g}$  LPS, and at this level of exposure, a rise in total cells and lymphocytes reached statistical significance. Available data on bronchial inflammation induced by inhalation of LPS in humans are limited. The major cellular response to more than 5  $\mu\text{g}$  LPS (or to dust containing LPS), reflected in the bronchoalveolar lavage fluid, are neutrophilia (1, 4, 26, 33), lymphocytosis (1, 4, 26) and increase in macrophages (26) and total cells (26, 33). However, both the increase in airway monocytes and decrease in macrophages induced by inhaled LPS are less known. The airway monocyte retention induced by LPS is consistent with *in vivo* animal model (34), whereas the decrease in sputum macrophages could be due to LPS-induced apoptosis of macrophages, as recently suggested *in vitro* in human alveolar macrophages (35).

In our subjects, the threshold value for no response in sputum inflammatory cells is 0.5  $\mu\text{g}$  inhaled LPS. This exposure level is corresponding to a subject exposed 10 h in a day and ventilating per hour 1  $\text{m}^3$  air containing 50 ng LPS. In occupational settings, endotoxin is frequently above 100 ng/ $\text{m}^3$  air, in particular in swine confinement buildings, poultry farms, cotton industries, and waste handling (7, 8, 10), and could lead to inflammation in airway diseases. In the domestic environment, endotoxin exposure is less intense since air and house dust are loaded by endotoxin < 1 ng/ $\text{m}^3$  (36) and < 1 ng/mg (9, 37), respectively. Dubin and colleagues have recently reported in allergic asthma that exposure to domestic allergen provokes a plasma exudation in the airways with extravasation of the LPS-binding protein (LBP) and sCD14, the soluble receptor for LBP-LPS complexes (38). That extravasation of LBP and sCD14 into the bronchoalveolar compartment may enhance by 100-fold the capacity of inhaled LPS to activate its inflammatory cascade, leading to airway disease (38). This suggests that, in allergen-exposed atopic subjects, the no-response threshold value of endotoxin exposure is probably far less than 0.5  $\mu\text{g}$  inhaled LPS.

The increase in MPO concentration in sputum is related to the dose of inhaled LPS. The MPO enzymatic system is released from the PMNs granules and directly involved in the production of oxygen radicals, leading to cell damage in the airways (39). This LPS-induced change in MPO is probably not due to activation of the airway PMNs but rather is related to PMN infiltration since both the PMNs and MPO responses are parallel. Contrary to the eosinophil count, the ECP concentration increases in sputum liquid, suggesting an involvement of eosinophils in the response to LPS. *In vitro*, a small amount (< 0.1 ng/ml) of LPS is able to enhance human eosinophil survival as well as release of several cytokines, this effect being inhibited by IL-10 (40). This last observation in combination with the present results suggest that inhaled LPS could modify eosinophilic inflammation. Finally, a rise in TNF- $\alpha$  concentration in sputum is significant 6 h after inhalation of 50  $\mu\text{g}$  LPS as yet reported in the bronchoalveolar lavage after similar exposure (4, 33). As TNF- $\alpha$  may be involved in airway in-

TABLE 3  
THRESHOLD VALUES OF ENDOTOXIN EXPOSURE TO INDUCE A  
SIGNIFICANT RESPONSE FOR EACH PARAMETER

0.5 $\mu\text{g}$	5 $\mu\text{g}$	50 $\mu\text{g}$
CL-PMN	Blood PMNs Blood CRP Sputum PMNs Sputum monocytes Sputum MPO	Symptoms Fever Urine CRP Sputum lymphocytes Sputum ECP Sputum TNF- $\alpha$

Definition of abbreviations: CL-PMN = luminol-enhanced chemiluminescence of isolated blood PMNs; CRP = C-reactive protein; PMNs = polymorphonuclear neutrophils; MPO = myeloperoxidase; ECP = eosinophil cationic protein; TNF- $\alpha$  = tumor necrosis factor- $\alpha$ .

flammation in chronic obstructive pulmonary diseases (11), it could have relevance in pulmonary clinical symptoms (32).

In conclusion, this study has shown for the first time in humans that the response to endotoxin is related to the inhaled dose. The most sensitive markers of the LPS response are the changes in sputum and blood PMN count with their level of activation (by luminol enhanced chemiluminescence) and in CRP blood concentration (Table 3). Clinical symptoms and lung function are not sensitive to assess the response to inhaled LPS. The threshold value of the no-response to LPS acute exposure is lower than 0.5  $\mu\text{g}$ , corresponding to 10 h exposure to less than 50 ng/ $\text{m}^3$  airborne LPS.

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